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This project has focused on the development of a reliable animal model of stress-induced changes in immune function and the role of stressor controllability in this process. Previous work on this project concentrated on the effects of inescapable shock on changes in in vitro measures of immune function such as mitogen stimulated lymphocyte proliferation and natural killer cell cytotoxicity. Results were quite disappointing and although inescapable shock did produce changes in these measures, the changes were not robust and repeatable. This year we shifted to a study of in vivo immune functioning with the hope that in vivo measures might reveal more reliable changes. We developed an ELISA assay that detected specific IgG antibody to keyhole limpet hemocyanin (KLH), an immunogenic but harmless protein. We developed a paradigm in which animals were immunized with KLH, then stressed in some fashion, and then antibody to KLH measured over an 8 week period. A variety of different stressors all proved to substantially reduce antibody production. One session of inescapable shock, three sessions of inescapable shock, inescapable shock (OVER

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delivered during the dark phase of the animals' day/night cycle, inescapable shock delivered during the light part of the cycle, and defeat in territorial aggression all suppressed antibody production. Thus alteration in *in vivo* antibody production seems to be a robust and repeatable change in immune function produced by stressors, and we are now in a position to study the psychological and physiological mechanisms responsible for such changes.

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The goal of this research program has been to study the psychological variables that determine the impact of stressors on immune function and the neural and neuroendocrine mediators of any such effects. The first step in this process had to be the development of an animal model in which robust, repeatable, and meaningful changes in immune function would be produced by some stress condition. This has been a frustrating enterprise. We began by exploring the effects of inescapable electric shock in rats on mitogen stimulated lymphocyte proliferation and natural killer cell cytotoxicity. This work has been described in previous Annual Reports. We manipulated a large number of variables (number of shocks, shock intensity, number of shock conditions, colony conditions, culture conditions, etc) but were simply unable to produce a change in proliferation or cytotoxicity that was repeatable in a regular and consistent fashion relative to appropriate control groups. Sometimes effects occurred and sometimes they did not. We have not been able to isolate the variable or variables responsible for determining when effects do and do not occur. Of course, yet more severe stress conditions and even tighter control of colony conditions might be able to produce robust changes in the measures which we examined, but one would have to question the validity of the resulting paradigm as a model.

Moreover, at a number of national meetings (e.g., the Workshop on Methods in Behavioral Immunology sponsored by ONR and held in Tuscon, Arizona) the consensus among cellular immunologists seemed to be that in vitro and non-specific assays such as mitogen stimulated lymphocyte proliferation might have little relevance for the question really being posed--whether host defense to a pathogen is altered. This is because it is difficult to make inferences about the overall in vivo functioning of the immune system from these measures. The immune system is composed of a complex cascade of interacting cells and processes. It is quite possible that some experimental manipulation might depress, for example, T-cell proliferation by 25 or 50 %, but the in vivo recognition and destruction of antigen might remain intact.

It is also possible that the problems which we have encountered in producing reliable changes are related to the sensitivity of mitogen stimulated lymphocyte proliferation to stress effects, rather than with a general lack of effect of inescapable shock or other stressors on immune function. First, mitogen proliferation is highly variable under the best of conditions, as others have noted. We have been concerned by the magnitude of variability both within and across shipments of animals. Detection of even large effects of stressor exposure may be difficult when superimposed on even larger individual differences that exist with regard to mitogen proliferation.

Second, the in vitro nature of this assay may produce difficulties. In vitro techniques of necessity entail removal of the effector cells from their neuroendocrine and neural environment. It is these neural and endocrine influences that are most likely to mediate any impact of a stressor on immune function, and in vitro assays tend to minimize such influences. Since the effector cells have been removed from their normal internal milieu, possible neural or neuroendocrine influences that mediate stress effects must be long lasting and be carried into the tissue culture environment in order to alter the immune measure. This may be particularly problematic for the typical mitogen proliferation assay since 3 days

of incubation are generally employed. The relation of in vitro assays to in vivo functioning of the immune system is not well understood.

Other difficulties also exist. For example, there are sampling biases associated with the collection of cells from a single site such as peripheral blood or spleen. Different subsets of cells might be sampled from one study to the next, thereby creating difficulty in replicating findings. Moreover, stress can alter the circadian pattern of corticosteroid secretion, possibly shifting the circadian pattern of subset availabilities at a particular site. This would lead to very different results depending on the exact timing of sample collection, again contributing to variability.

For these reasons we changed our approach towards the measurement of an in vivo natural end-product of the integrated immune response, the generation of antibody to an antigen. We wanted to use an antigen that was harmless, that was highly immunogenic, and for which there were published protocols for antibody measurement. Keyhole limpet hemocyanin (KLH) met these requirements. We began by modifying an enzyme-linked immunosorbant assay and adapted it to the measurement of IgG to KLH. This assay has proved to be both highly sensitive and specific to KLH antibody. Briefly, the wells of a certified microtiter plate (NUNC, Immunoplate I) are coated with KLH. Plasma is serially diluted in the wells of these plates for each blood sample (1:500 to 1:64,000) and incubated for 3 hr at 37° C in a CO_2 incubator. Plates are washed and goat antirat IgG (Alkaline phosphatase conjugated) is added to each well and incubated for one hr at 37° C in a CO_2 incubator. Plates are washed again and substrate for the alkaline phosphatase is added. The color is allowed to develop for 15 min and the reaction is stopped by the addition of NaOH. Plates are read at 410 nm on the plate reader.

Inescapable Shock

We began with a series of experiments designed to determine whether exposure to inescapable shock would alter the generation of antibody to KLH. On Week 1 animals were immunized with KLH [0.2 ml KLH suspension (5 mg/ml sterile saline) injected at the base of the tail] and then immediately received a shock or control treatment. On Week 2 animals were again immunized with KLH and again immediately received a shock or control treatment. Animals were never shocked again. Blood samples were obtained from the tail vein on Weeks 2, 3, and 4. Blood samples were again obtained on Week 9, followed by another immunization with KLH. Blood samples were again obtained on Weeks 10 and 11. Because of our concern with the generalizability and repeatability of any results obtained we used 4 different shock conditions--either 1 or 3 daily sessions of inescapable shock following immunization on Weeks 1 and 2, with immunization and shock occurring in either the dark or the light part of the rat's day/night cycle. These variables were chosen because the chronicity of stress and when it occurs during the circadian rhythm are known to be important modulators of stress effects. Control treatment consisted of removing subjects from their cages, bringing them up to the laboratory, and treating them exactly the same as the experimental subjects except that no shock was delivered. This is an important point because many experiments have often compared experimental groups to only home cage controls. Thus any difference between groups could be attributable to handling, exposure of the subjects to novel environments and pathogens, etc, rather than the specific stressor used.

In sum, the experiment investigated 1 versus 3 sessions of shock following each of the two primary immunizations, during the light or during the dark. Blood samples were obtained from the tail vein using a standard procedure in our laboratory that requires very little disturbance of the rat. This was done 1, 2, 3, 8, 9, and 10 weeks following the first

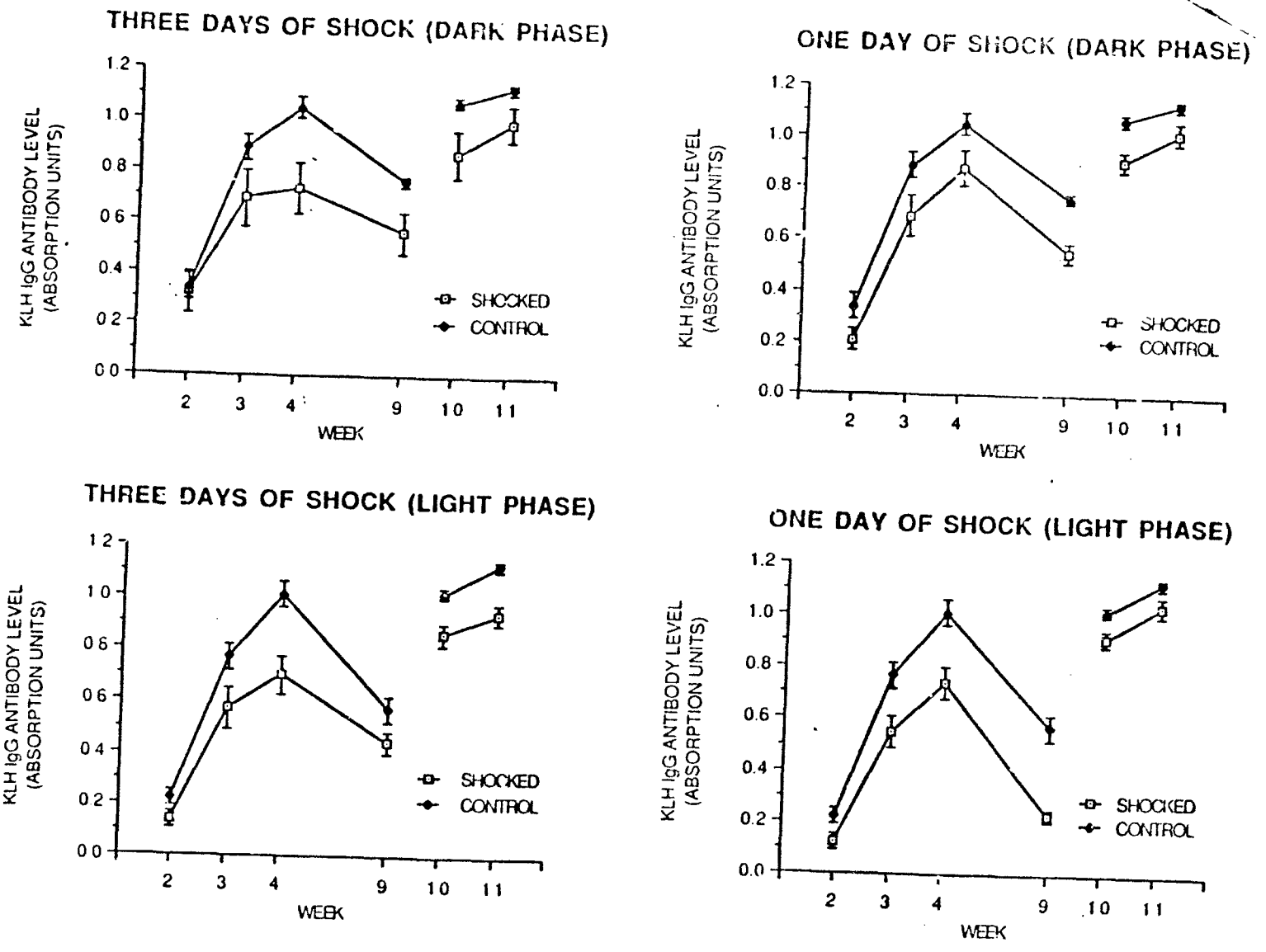
THREE DAYS OF SHOCK (DARK PHASE)
KLH IgG ANTIBODY LEVEL (ABSORPTION UNITS)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
3
4
9
10
11
WEEK
SHOCKED
CONTROL
ONE DAY OF SHOCK (DARK PHASE)
KLH IgG ANTIBODY LEVEL (ABSORPTION UNITS)
WEEK
SHOCKED
CONTROL
THREE DAYS OF SHOCK (LIGHT PHASE)
KLH IgG ANTIBODY LEVEL (ABSORPTION UNITS)
WEEK
SHOCKED
CONTROL
ONE DAY OF SHOCK (LIGHT PHASE)
KLH IgG ANTIBODY LEVEL (ABSORPTION UNITS)
WEEK
SHOCKED
CONTROL

Figure 1

immunization with KLH. Thus, all groups received 3 immunizations with KLH--on Days 1,8, and 57 of the experiment. Antibodies were determined for samples taken on Days 8, 15, 22, 57, 64, and 71, referred to as Weeks 2, 3, 4, 9, 10, and 11. Shock sessions were conducted on Days 1 and 8 for one group and on Days 1,2,3,8,9, and 10 for a second group.

The results for the 4 conditions and controls for each can be seen in Figure 1. The data presented here are KLH IgG antibody levels (absorption units) for a 1:1000 serum dilution. Comparisons are never made across "runs" of the assay. Examining the data for the primary antibody response, it is evident that the response to KLH increased across the first 3 weeks and declined by Week 9. Comparisons across figures suggests that antibody levels are greater if immunization occurs during the dark phase of the cycle. It is also apparent that each of the 4 shock conditions (1 and 3 sessions occurring in the day or night) reduced the development of antibody to KLH to a roughly equal degree. This effect increased as antibody level rose. Recall that the last immunization and shock sessions occurred during Week 2. Thus the difference in antibody levels at Week 4 reflects a difference remaining 2 weeks after the last experimental treatment. Moreover, the impact of the shock exposure was still evident 7 weeks after the last shock experience. These conclusions are strongly confirmed statistically, but space precludes inclusion here. Further, these differences remain reliable at all dilutions from 1:200 to 1:12,800, beyond which the assay cannot readably detect antibody. The results for Weeks 10 and 11 were equally impressive. However, interpretation of this data is complicated by the fact that the primary response had not totally declined. Thus the data here is a mixture of primary and secondary response.

Because of our concern with repeatability, generality, and desire for systematic parametric information we have gone on to conduct a variety of further studies. A dose-response study indicated that our dose of KLH was quite high (making the above results all the more impressive). We have now used a much smaller dose (0.05 ml), a single immunization, and a single session of inescapable shock. The results are superimposable on those shown in Figure 1. The fact that we can obtain our effect with a single session of shock following a single immunization with KLH makes exploration of factors such as antigen-stress timing much simpler. We are currently exploring shock parameters in order to determine the minimum necessary to produce an alteration in KLH antibody production.

Defeat

Others have noted several negative features of using inescapable shock as a stressor. First, organisms do not encounter inescapable shock in the "real world", and it would be desirable to develop a model using a more "ecologically valid" stressor. Second, human stressors tend to involve psychosocial conditions in which the individuals appraisal of the situation is a critical element, rather than physically painful stimulation. It would thus be desirable to have an animal model which mimics this element. Here the influence of the subject's reaction to the situation would be separable from primary physical reactions to the events, such as pain and nausea . This sort of model might also have an additional advantage to the presentation of directly painful events such as electric shock. There are always large individual differences in how a group of subjects exposed to a stressor react to the stressor events, and this is certainly true of immune system changes. It might well be that some aspect of how the individual behaves in reaction to the stressor might predict the degree of effect that the stressor would have on whatever outcome is of interest, such as immune function. However, most of the paradigms in which an organism is exposed to a simple physical stressor such as electric shock, immobilization, etc, do not lend themselves to measurement of individual behavioral reactions.

For a number of reasons defeat in aggressive encounters seemed to be a paradigm which might meet these conditions. We developed a colony-intruder paradigm in our laboratory as a model of territorial aggression. In this paradigm 2 males are allowed to live in the same environment (a large tub cage) for several months. One of the males will become dominant during that time. An "intruder" is then introduced. The dominant male will attack the intruder with a fairly short latency and the intruder is removed after 10 min. During this period attack will usually have occurred and it is ordinarily enough to induce submissive posturing in the intruder. The intruder initially retaliates, then engages in defensive reactions, such as the defensive upright posture and escape attempts. Eventually the intruder displays a submissive response pattern characterized by upright body posture with raised limp forepaws, upwardly angled head, and retracted ears. Frequently the animal will turn over on its back and expose its ventral surface. Once defeated, rats show these behavioral features even before being attacked and fail to orient toward the opponent. The intruder can thus be returned for any number of sessions while producing little if any bites or physical injury. Many details of the procedure are crucial to producing reliable aggression in the resident and defeat in the intruder (e.g., resident males must be quite large, sessions must be conducted during the dark part of the residents cycle, colony diads must have lived together for several months and have experienced previous intruders since colonies become more aggressive with experience, etc). We have now had sufficient experience with this procedure so that we can produce very reliable aggression and defeat of the intruder. With our procedures the intruder invariably "gives up", probably because it "knows" it is in another male's territory via odor cues. We videotape the encounters and have developed a very reliable scoring procedure (we count number of bites, latency to adopting a defeat posture, amount of time spent in submissive postures, etc.) with inter-rater reliabilities of over .93.

Our first experiment was modeled after the shock study. Rats (N=12) were immunized with KLH on Days 1 and 8 and exposed to 50 min of defeat experience after each of the immunizations. Fifty min of defeat was arranged by successively exposing the rats to 5 different aggressive colonies, each for 10 min. Simply allowing an intruder to remain in a single colony for 50 min will not produce sustained submissive posturing or aggression. Nor will a given colony aggress again immediately after an intruder has been present. A given resident colony can only be used once a day, and so many colonies must be available. Control rats (N=12) were exposed to successive nonaggressive colonies for an equal period of time. Thus these animals were moved, placed in the presence of unfamiliar conspecifcs, etc, but were not attacked and therefore did not submit. Blood samples were taken on Weeks 2, 3, and 4 and serum IgG antibodies to KLH determined.

The introduction of the intruder into resident colonies depressed antibody production measured several weeks later. This defeat procedure has the advantage over the inescapable shock procedure that we have a variety of behavioral measures (e.g., bites, submission latency, total submission time during the 50 min, etc.) on the animals and so can determine whether any predict the magnitude of antibody production. That is, we can determine whether the behavior or experience of the subjects during the defeat episode might account for individual differences in the magnitude of the effect. We examined simple correlations. partial correlations, and performed stepwise multiple regressions using a large number of predictor variables. The interesting result of each of these ways of examining the data was that bites did not predict antibody production ($r = .11$). Indeed, there were cases of rats which did not receive a single bite or even physical contact, but showed low antibody production. By far the best predictor was time spent in submissive postures ($r = .68$). Indeed the r^2 between submission time and antibody level partialling out bites was .71. Thus an incredibly high percentage of the variance in antibody

production in the animals subjected to the defeat experience was accounted for by time spent in defeat posture.

As with the shock situation we have been very concerned to determine whether the defeat effect is repeatable and general. As with the shock procedure we now know that a single immunization followed by a single 50 min defeat experience is sufficient to produce a robust effect. We do not yet know precisely how much defeat is required, but a single 10 min experience is not sufficient.

In sum, we are now very confident that the two stressors in which we are interested will produce a reduction in antibody generation to the antigen KLH and will do so across a range of conditions and parameters. These results, we believe, justify further systematic study.

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